control and 0.332 in the supernatant. The control numbers in the above experiments refer to the analyte concentration measure before extraction, where 300 μL of water was used instead of the particles.

REMARKS

Minor amendments have been made to the Specification to correct obvious typographical errors and to update prior application data. The amendments to the Specification do not add any new matter to the application or affect the claimed invention.

It is believed that no additional fee is due with this Amendment. If this is in error, please charge any necessary fees to Deposit Account No. 19-5117.

Respectfully submitted,

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Marked up version showing changes to specification under 37 C.F.R. § 1.121(b)(iii)

Paragraph 6

Specific affinity binding is a technique used to capture specific target ligands from complex mixtures such as biological fluids. For example, monoclonal or polyclonal antibodies may be immobilized on a surface. When the surface is contacted with the sample, the antibodies bind to components of the mixture. Analysis is conventionally carried out via competitive binding, or in a "sandwich" assay using a secondary antibody. In both modes, there is usually a tag (enzyme, radiolabel, fluorophore, etc.) that is used for detection and/or amplification. An alternative approach is direct detection of bound analytes by surface plasmon resonance or quartz crystal microgravimetry. Specific affinity binding techniques have been applied to proteomics in order to characterize gene products. Although it is highly specific, such immunoseparation has many of the same drawbacks as other assays that take place in two dimensions. Moreover, immunoseparation fails when there is no high-affinity antibody available ~~to components~~ a desired component in the sample, which is often the case. In particular, immunoseparation provides unsatisfactory results with respect to (i) unknown molecules, (ii) known protein molecules that are post-translationally modified at or near the high affinity epitope; and (iii) molecules too small to elicit a strong immune response.

Paragraph 7

One specific affinity binding approach to proteomics where the analysis is limited to known proteins (i.e., proteins for which antibodies are commercially available) is the state-of the-art FlowMetrix® system developed and commercialized by Luminex™ Corp. (Austin, Texas). The FlowMetrix® system uses microspheres as the solid support for performing multiplexed immunoassays. Currently Luminex™ offers 64 different bead sets. Each bead set can, in principle, support a separate immunoassay and the beads are read using an instrument similar to a conventional flow cytometer. A major limitation of the Luminex™ approach is that the frequency space of molecular fluorescence used both for microsphere tagging and detection is not wide enough to accommodate nearly as many different assays as would be desirable to fully realize the advantages of multiplexing.

Paragraph 18

Two alternative technologies with potential relevance both to combinatorial chemistry and genetic analysis involve "self-encoded beads", in which a spectrally identifiable bead substitutes for a spatially defined position on a solid supporting chip. In the approach pioneered by Walt and co-workers, beads are chemically modified with a ratio of fluorescent dyes intended to uniquely identify the beads, which are then further modified with a unique chemistry (e.g., a different antibody or enzyme). The beads are then randomly dispersed on an etched fiber array so that one bead associates with each fiber. The identity of the bead is ascertained by its fluorescence readout, and analytes are detected by fluorescence readout at the same fiber in a different spectral region. The seminal reference (Michael *et al.*, Anal. Chem., 70, 1242-1248 (1998)) describing this technology suggests that with 6 different dyes (15 combinations of pairs) and with 10 different ratios of dyes, 150 "unique optical signatures" could be generated, each representing a different bead "flavor." A very similar strategy is used by Luminex™ that combines flavored beads ready for chemical modification with a flow cytometry-like analysis. (See, e.g., McDade *et al.*, Med. Rev. Diag. Indust., 19, 75-82 (1997)). Luminex™ states that its self-encoded beads enable researchers to assay up to 100 analytes in a single sample. The particle flavor is determined by fluorescence and, once the biochemistry is put onto the bead, any spectrally distinct fluorescence generated due to the presence of analyte can be detected. As currently configured, it is necessary to use one color of laser to interrogate the particle flavor, and another, separate laser to excite the bioassay fluorophores.

Paragraph 21

SPME followed by CE as the second dimension has been used to analyze a mixture of peptides from a proteolytic digest. (~~Yates~~ Tong, Anal. Chem., 71, 2270-2278 (1999)). Although the SPME-CE/MS improved the concentration detection limit by more than two orders of magnitude when compared to CE-MS alone, the large electro-osmotic force of the aminopropylsilane (APS) coated capillary tended to elute all the

peptides in a relatively short period of time. This presents the possibility of confounding results owing to the co-elution of compounds.

Paragraph 22

A strategy has been used for the separation of MHC class I peptides, several thousand such peptides exist at sub-femtomolar concentrations. The literature reports immuno-affinity concentration followed by reverse phase, and subsequent concentration on specially designed membranes capillaries. (Naylor, Chromatogr., 744, 237-78 (1996)). In addition, a comprehensive two-dimensional separation technique has been described for profiling proteins. (~~Jorgenson~~ Opiteck *et al.*, Anal. Chem., 69, 1518-1524 (1997)).

Paragraph 45

The concentration of analytes via extraction into the extraction phase serves as a discrete separation process. In addition to the types of extraction phases and solid supports empolyed, the extraction can be manipulated by changing the reaction conditions, such as the temperature, pH, pressure, concentration, and ionic strength. In addition, the exposure time may be varied. Some analytes can be expected to require more time than others to partition into the extraction phase. In many cases, it is impracticable to wait for equilibrium to be reached. Rather, the extraction probes can be exposed to the sample for a given length of time, ~~and extract the~~ extracting a particular amount of analyte in that time. By varying these conditions, additional information can be obtained from the sample. For instance, using the hypothetical above, assume that the analytes extracted by the probes changes as a function of pH. Thus, at a higher pH, probe III might extract a third analyte, Analyte D. Thus, changing the pH would allow additional information to be obtained using the same set of extraction probes.

Paragraph 63

Solid phase extraction arrays, such as protein chips, rely on the physical position on the array to identify the matrix of captured analytes on the chip surface. Thus, the nature of the associated extraction phase, for example, can be readily identified by the

spatial address of the extraction phase. Such two-dimensional arrays enable assays of multiple analytes to be conducted in parallel. However, slow diffusion of analytes to the planar surface limits ~~its~~ their application. Encoded particle-based extraction probes of the present invention provide an alternative approach ~~to~~ in which there is a three-dimensional array in solution ("solution arrays"). Substituting for the spatially defined position on a solid supporting chip array, the encoded information allows individual elements to be addressed when positional information has been eliminated by random distribution in space. This approach retains the advantages of two-dimensional arrays for massively parallel analyses with diverse extraction probes at high throughput, but does not compromise on the kinetics of binding in solution. Furthermore, because each element in a solution array is independent, there is flexibility to interrogate a few or thousands of analytes without the need to fabricate a new chip with a custom set of extraction phases. In addition, a spatially defined position may be introduced to the system of encoded particle-based extraction probes, e.g., after contact with the sample, resulting in an "array of arrays." This may be accomplished, for example, by sorting the probes into spatially defined areas of a two-dimensional surface; typically, into wells of a microtiter plate. The sorting may occur either before or after contacting the probes with the sample and may be effected by any of a number of means known in the art (e.g., in flow). Reintroducing a spatially defined position will allow the extraction probes to be interrogated in the same ways currently used for two-dimensional arrays (e.g., protein chips). In those embodiments in which a spatially defined position is introduced, it adds another level of multiplexing to the assay – for example, a set of 104 differentiable extraction probes distributed in each well of a 96-well plate will effectively create nearly 10,000 individually addressable extraction probes, and allow nearly 10,000 of extraction phases to be contacted with sample. In this way, using differentiable extraction probes with two dimensional arrays would decrease the cost and time required to obtain useful information, because a smaller set of differentiable solid phases would have to be synthesized.

The table between Paragraph 73 and Paragraph 74

LIGAND	COUNTERLIGAND
Cofactors	Enzymes
Lectins	Polysaccharides, glycoproteins
Nucleic acid	Nucleic acid binding protein (enzyme or histone)
Biomimetic dyes	Kinases, phosphatases, Dehydrogenases etc..
Protein A, Protein G	Immunoglobulins
~~Metals~~ Metal ions	Most proteins can form complexes with metal ions
Enzymes	Substrate, substrate analogues, inhibitor, cofactors
Phage displays	Proteins, peptides, any type of protein
DNA libraries	Complementary DNA
Aptamers	Proteins, peptides, any type of protein
Antibody libraries	Any type of protein
Carbohydrates	Lectins
ATP	Kinases
NAD	Dehydrogenases
Benzamide	Serine Protease
Phenylboronic acid	Glycoproteins
Heparin	Coagulation proteins and other plasma proteins
Receptor	Ligand
Antibody	Virus

Paragraph 88

Although the above discussion has focused on the highly preferred segmented-nanoparticle extraction probes, one of skill in the art will recognize that other encodeable nanoparticles could be used according to the principles of the invention. For example, in one embodiment of the invention the particle solid supports are not comprised of segments, but are differentiable based on their size, shape or composition. Such an array of particles, which can be made up of any material, is comprised of at least 2, preferably at least 3, and most preferably at least 5 types of particles, wherein each type of particle is differentiable from each other type of particle. In the preferred embodiment, since the types of particles may be comprised of a single material and since different types of particles may be comprised of the same material as other types of particles in the assembly, differentiation between the types is based on the size or shape of the particle types. For example, an assembly of particles of the present invention may be comprised of 5 different types of gold rod-shaped nanoparticles. Although~~,~~ each type of rod-shaped particle ~~have~~ has a roughly similar ~~widths~~ width or ~~diameters~~ diameter, the different types

of particles may be differentiable based on their length. In another example, 7 types of spherical silver particles make up an assembly. The different types of particles are differentiable based on their relative size. In yet another example, 8 types of rod-shaped particles, all composed of the same polymeric material, make up an assembly; although each type of rod-shaped ~~particles have~~ particle has the same length, they are differentiable based on their diameter and/or cross-sectional shape.

Paragraph 103

The segments may be comprised of any material. In preferred embodiments of the present invention, the segments comprise a metal (e.g., silver, gold, copper, nickel, palladium, platinum, cobalt, rhodium, iridium); any metal chalcognide; a metal oxide (e.g., cupric oxide, titanium dioxide); a metal sulfide; a metal selenide; a metal telluride; a metal alloy; a metal nitride; a metal phosphide; a metal antimonide; a semiconductor; or a semi-metal. A segment may also be comprised of an organic mono- or bilayer such as a molecular film. For example, monolayers of organic molecules or self-assembled, controlled layers of molecules can be associated with a variety of metal surfaces.

Paragraph 108

A segment may be comprised of any organic compound or material, or inorganic compound or material or organic polymeric materials, including the large body of mono and copolymers known to those skilled in the art. Biological polymers, such as peptides, oligonucleotides and ~~carbohydrides~~ carbohydrates may also be the major components of a segment. Segments may be comprised of particulate materials, e.g., metals, metal oxide or organic particulate materials; or composite materials, e.g., metal in polyacrylamide, dye in polymeric material, porous metals. The segments of the nanoparticles used in the present invention may be comprised of polymeric materials, crystalline or non-crystalline materials, amorphous materials or glasses.

Paragraph 120

For example, DNA may be separated from salts using C18 resin as the extraction phase as follows. First, a 96-well array of C18 coated needles is washed three times with

Acetonitrile and then equilibrated in 50 mM triethylammonium acetate (TEAA) buffer, pH 6.5. Next, the array is soaked in a mixture of DNA and salts, allowing the DNA to bind to the C18 resin. Then the array is removed from the mixture and washed three times in the equilibration buffer (TEAA) to remove the unbound salts. Finally, the bound DNA is extracted into 50% ~~Acetonitrile~~ acetonitrile.

Paragraph 127

The present invention allows several methods for solid phase micro- or nanoextraction of analytes in parallel. For example, chromatographic media could be placed as a micro column within a pipette tip called a ~~Zip-Tip~~ ZipTip® (Millipore®) or, alternatively, coated on the inner surface of a pipette tip such as Supro-Tip and Pro-Tip (Amika Corporation/Harvard Apparatus). These are hollow objects with chromatographic media coated or as a plug in the hollow structure. A multichannel pipettor would allow parallel processing using these tips.

Paragraph 133

Another advantage to using a bead-based extraction probe is the ability to access samples that would otherwise be difficult to obtain. For example, if one wanted to carry out solid phase micro- or nanoextraction on whole blood in circulation, one could use beads smaller than the diameters of capillaries. Alternatively, larger solid support beads could also be used if the animal were to be sacrificed at the end of the experiment, or if the bead extraction probes could be localized within certain body compartments (either natural or artificially created). To collect these beads, one could make them magnetic, thus allowing them to be readily removed from an organism after a certain time. Beads can be made magnetic by incorporation of magnetic material on the interior, exterior, or both. Magnetic retrieval allows the bead extraction probes to be isolated with minimal sample perturbation, for example, in applications where centrifugation is disfavored (i.e., in whole blood). Of course, numerous alternative methods of bead retrieval are available, including without limitation, centrifugation, gravity-based particle settling (in a non-gradient containing or gradient containing column, or even in solution), optical methods (e.g., optical trapping), and bead based flow/sorting methods (e.g., using cytometry and

fluorescence-activated cell sorting (FACS)). In addition, bead-based extraction probes may be collected using microfluidic devices by one of a number of different methods, including isoelectric focusing, dielectrophoresis, acoustic focusing, among a number of others. In addition to being collected, particles can also be sorted by dielectrophoretic trapping (see, e.g., Cummings & Singh, *in* Microfluidic Devices and Systems III, Proc. SPIE, 4177, 164-173 (2000)) or by a number of analogous methods.

Paragraph 150

There are alternatives to using SAMs. For example, nanoparticles could be coated with polymers; the polymers could be synthetic organic polymers. Each polymer coat serving as an extraction phase can have selected properties. Monomers may be attached to the nanoparticles and the polymerization reaction conducted directly on the surface of the nanoparticle. (E.g., Mirkin, ~~WO 99-U/S28387~~ WO 99/28387, "Preparation of Nanoparticles with Polymer Shells for Use in Assays.").

Paragraph 152

Each extraction phase could be uniquely designed to capture only one class of molecules. Such classes may include large molecules, such as proteins. In addition, combinatorially prepared extraction phases could be empirically tested to determine which molecules present in a sample were captured, and to what extent. If the desired criteria can be specified, such screening could be automated to take place in a high-throughput manner to determine the appropriate extraction phases that meet the desired criteria. For example, it may be that the presence of a certain metabolite is suspected of being a significant marker for a disease. Thousands of extraction probes could be prepared by coupling encoded particles (e.g., segmented-nanoparticles) with combinatorially prepared extraction phases, the code specifying the extraction phase or the method for its preparation. These extraction probes could then be ~~screend~~ screened against a sample known to contain the metabolite. The extraction probe, or set of extraction probes, found to best extract the metabolite (e.g., as assayed by mass spectrometry) could be determined from the encoded information and used subsequently to assay unknown samples. This same empirical method could be used to arrive at a set

of extraction probes that produces a meaningful fingerprint of a sample. This would decrease the cost and time required to obtain useful information, because only the minimal number of probes that completely describe the sample would have to be synthesized and analyzed. Thus, rather than contact the sample with an arbitrarily large number of unique extraction probes, a limited set (e.g., <50) could be used that have been found to pan the sample. The desirable set of extraction probes may be different for different samples. Thus, the set of extraction probes desirable for panning urine of diabetic patients would likely be different from the set of extraction probes desirable for panning synovial fluid from an arthritic patient.

Paragraph 173

The supernatant was discarded and the particles were subjected to a final resuspension, centrifugation followed by a final suspension in 100μL of phosphate buffer (pH 8.0. 100mM). A solution of succinic anhydride in DMSO (100mg/mL) was prepared and added dropwise to the suspension of the dextran coated segmented-nanoparticles. The succinic anhydride solution was added in 10μL aliquots followed by 10μL aliquots of 1.0(M) NaOH between each addition. A total of 10 additions were made over a period of 30 minutes. The particles were washed by the usual centrifugation, removal of supernatant followed by resuspension for a total of 3x1mL washes. The carboxy-terminated dextran coated segmented-nanoparticles were then stored in 100μL of water. The presence of dextran was qualitatively determined by the anthrone test (Jermyn, Anal. Biochem, 68, 332-335 (1975)).

Paragraph 179

The oligonucleotide particles were prepared by incubating biotinylated (dT)21 mer (0.3 nmoles, HPLC purified from IDT Inc) with streptavidinated particles ($2x10^8$ particles, $1x10^5$ streptavidin per particle) in a TRIS/EDTA buffer (100μL, pH 8.0). The particles and the oligonucleotides were shaken in an end over end shaker for 1h, after which ~~it was~~ they were washed by centrifugation, removal of supernatant and resuspension in water for a total of three cycles.

Paragraph 182

NHS-Ester linked beads (10mg) were taken up in pH 9, TAPS buffer in a 1.5ml eppendorf® tube. To the bead solution, 20 µl of aminophenylboronic acid (10mg/ml) was added. The solution was rotated overnight. The beads were then centrifuged and the supernatant removed. The beads were then resuspended in fresh TAPS buffer and washed two more times before they were suspended to a final concentration of 50mg/ml in TAPS buffer.

Paragraph 192

Biotin-Flourescein Conjugate Depletion Was Determined By Fluorescence (490 Excitation, 520 Emission) 58597 Units For Control And 3369 In Supernatant. Glucose depletion was determined by the anthrone test, (Anal. Biochem, 68, 332-335 (1975)). ~~absorbance~~ Absorbance at 626nm was 0.121 for control and 0.06 in the supernatant. Diamine depletion was determined by the TNBS assay;. ~~absorbance~~ Absorbance at 415 nm was 0.620 for control and 0.332 in the supernatant. The control numbers in the above experiments refer to the analyte concentration measure before extraction, where 300 µL of water was used instead of the particles.

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